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Expression of EVI1 and the Retinoblastoma Genes in Acute Myelogenous Leukemia With t(3;13)(q26;q13–14)

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The EVI1 DNA-binding protein gene on chromosome 3q26 has been reported to be activated in some leukemia cells with alterations in 3q26. We present an acute myelogenous leukemia (AML) patient with a rare chromosomal translocation, t(3;13)(q26.2;q13–14). By reverse transcription- polymerase chain reaction, we detected active transcription of the EVI1 gene in the patient's leukemia cells. The retinoblastoma susceptibility (Rb) gene, a tumor-suppressor gene, is located at chromosome 13q14 and is within the other translocation breakpoint in this patient. The expression of the Rb gene product was found to be substantially decreased in the patient's leukemia cells by Western blotting. Southern blot analysis, however, revealed no gross abnormalities of the Rb gene. Although it is unlikely that the Rb gene is directly involved in this translocation, the loss of the Rb gene product combined with the activation of the EVI1 gene may have led to the development of leukemia. © 1996 Wiley-Liss, Inc.

Key words: chromosomal translocation, chromosome 3, chromosome 13, EVI1 gene, Rb gene

INTRODUCTION

The EVI1 gene, a putative oncogene which maps to human chromosome 3q24–28, encodes a member of the DNA-binding zinc finger family of transcriptional factors [1,2]. Originally discovered as a site of retrovirus insertion in murine myeloid leukemias, the EVI1 gene has since been implicated in leukemogenesis [3]. Previous studies have shown that the aberrant expression of the EVI1 gene blocks the differentiation of myeloid or erythroid progenitor cells [4,5].

The EVI1 gene recently was shown to be transcriptionally activated in human acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), and chronic myelogenous leukemia (CML) in blast crisis, with rearrangements of chromosome 3 involving band q26 [6–9]. In leukemia cells with t(3;21)(q26;q22), the EVI1 gene was found to be fused to the AML1 gene [10,11], which is involved in AML with t(8;21)(q22;q22) [12].

We present a patient with AML and a new chromosomal translocation, t(3;13)(q26.2;q13–14). The retinoblastoma susceptibility (Rb) gene, a tumor-suppressor gene, maps to chromosome 13q14 [13]. In addition to various types of solid tumors, the Rb gene product has been shown to be substantially reduced in a subset of cases of AML [14,15]. Considering the possibility of the interaction between the EVI1 and Rb genes, we analyzed the expression of the two genes in the leukemia cells of this patient.

CASE REPORT

A 53-year-old Japanese man was referred to our hospital with thrombocytopenia and leukocytosis on August 3, 1992. There were numerous petechiae over his body, including his extremities. A complete blood count revealed a hemoglobin concentration of 7.4 g/dl; a leukocyte count of 225.0×10^9/l with 97% blasts, 2% neutrophils, and 1% lymphocytes; and a platelet count of 57×10^9/l, with a normal size distribution of platelets. The blasts were immature, without cytoplasmic granules,

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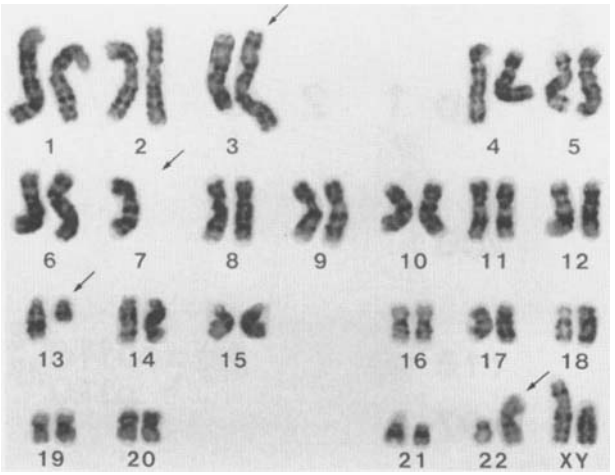

Fig. 1. The karyotype, 45,XY,t(3;13)(q26.2;q13–14),−7, idic(22)(p11), of the peripheral blood leukemia cells.

and were positive for myeloperoxidase staining. The serum blood chemistry showed a high level of lactate dehydrogenase. The blasts from the peripheral blood were positive for CD5 (24.5%), CD7 (25.3%), CD13 (98.4%), CD33 (24.4%), CD34 (82.9%), and HLA-DR (92.9%), and were negative for CD2, CD3, CD4, CD8, CD10, CD14, CD16, CD19, CD20, CD25, and CD56. A diagnosis of CD7-positive AML was made. Chemotherapy consisting of a combination of daunorubicin and cytosine arabinoside was begun on day of admission. However, the patient died of acute cardiac failure the next day, before a bone-marrow examination could be performed. Therefore, his leukemia could not be classified according to the French-American-British (FAB) classification.

MATERIALS AND METHODS

Cells

Two types of leukemia cells were used as positive controls for EVI1 gene expression: one was from an AML patient with t(3;3)(q21;26), and the other was from an AML patient with inv(3)(q21q26). The former has been described in detail previously [16]. Leukemia cells with these abnormalities of chromosome 3 have been reported to show activation of the EVI1 gene [6,7,9]. RM10, a Philadelphia chromosome-positive erythroid leukemia cell line [17], with no abnormalities of chromosome 3, was used as negative control.

For detection of the Rb gene product, Daudi, a Burkitt lymphoma cell line, was used as positive control [18]. The Daudi cell line was provided by the Japanese Cancer Research Resources Bank (Tokyo, Japan). In all clinical samples, the mononuclear cells were separated by Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) centrifugation before use.

Cell Culture and Establishment of a Lymphoblastoid B-Cell Line

The patient's peripheral blood mononuclear cells obtained at diagnosis were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum in 16-mm wells. Gradual proliferation of cells with cluster formation was noted about 2 months after initiation of cell culture. The cells were positive for CD19, CD20, and HLA-DR, and negative for myeloid markers. Chromosomal analysis showed a normal karyotype. The cells were maintained in continuous culture for more than 6 months. Infection with Epstein-Barr virus in this cell line was not determined.

Detection of EVI1 mRNA by Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

RT-PCR analysis for detection of EVI1 mRNA was performed as previously described by Morishita et al. [8], with the following modifications. Total RNA was extracted from 1×10^6 leukemia cells with hot phenol, and subsequently with phenol-chloroform. cDNA was synthesized from RNA by reverse transcriptase reaction with RAV-2 reverse transcriptase (Takara, Kyoto, Japan) [19]. The cDNA solution was mixed with PCR buffer, dNTP, sense primer (5′-AGCAACGTCGAATCAAGACCTGCTTGAGAT-3′), antisense primer (5′-ACTGACTGTAAGAGCTCACTGGCCTCAGGT-3′), and Taq DNA polymerase (Promega, Madison, WI). PCR was performed for 30 cycles, with each cycle consisting of 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C.

PCR products were subjected to electrophoresis and stained with ethidium bromide. The identity of the EVI1 gene transcripts was confirmed by Southern blotting. Southern blots were probed with a ^{32}P end-labeled oligonucleotide (5′-AGGGCACTGAAGCTCTCTAGCTTTTCTGCC-3′), which recognizes bases 2328–2357 internal to the primer sequences [2].

Western Blot Analysis of Rb Gene Product

Cells were lysed with lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecylsulfate (SDS), 1% sodium deoxycholate, 0.5 M EDTA, 0.3 M phenylmethylsulfonyl fluoride, 250 U/ml aprotinin, 20 mM sodium fluoride, 100 μM sodium orthovanadate, and 50 μg/ml leupeptin). Equal amounts (50 μg) of protein in each lysate were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter. The filter was preincubated overnight with blocking buffer and then incubated with a mouse monoclonal antibody against the human Rb gene product (PMG3-245, Pharmingen, San Diego, CA). After extensive washing, the filter was incubated with an anti-mouse immunoglobulin antibody con-

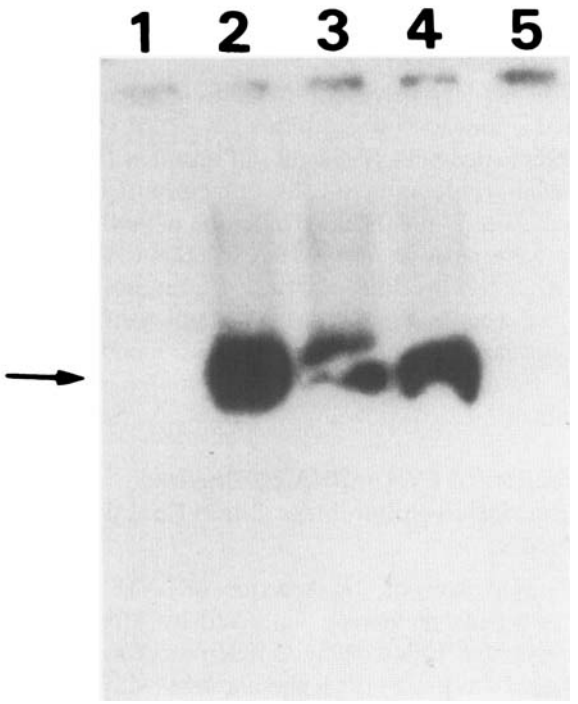

Fig. 2. RT-PCR of EVI1 gene transcripts followed by Southern blot using an oligonucleotide probe specific for the EVI1 gene. Lane 1, RT-PCR product from RM10 cells; lane 2, RT-PCR product from present case; lane 3, RT-PCR product from inv(3)(q21q26) cells; lane 4, RT-PCR product from t(3;3)(q21;q26) cells; lane 5, blank control. Arrow indicates 280-bp EVI1 transcript.

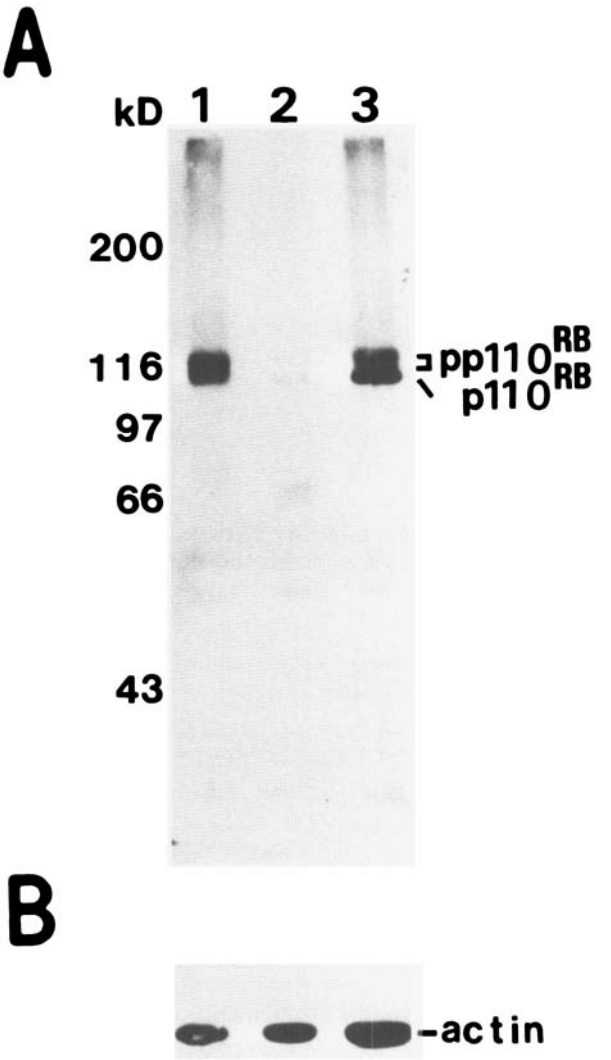

Fig. 3. A: Western blot analysis of Rb gene product. Lane 1, lysate from Daudi cells; lane 2, lysate from present case; lane 3, lysate from lymphoblastoid B-cell line established from the patient's peripheral blood. Phosphorylated 115-kD and unphosphorylated 110-kD Rb proteins are indicated. Molecular weight markers appear at left. B: Western blot analysis of actin. The same filter as in A was incubated again with an anti-actin antibody. No protein degradation of actin was seen.

jugated with horseradish peroxidase. It then was exposed to a chemiluminescence mixture and visualized according to the directions of the manufacturer (Amersham, Buckinghamshire, UK). The filter was reprobed with mouse anti-actin antibody (Amersham) to check the integrity of the proteins in the lysate.

Southern Blot Analysis of Rb Gene

DNA extraction, digestion with restriction enzymes, agarose gel electrophoresis, Southern blotting, and hybridization were preformed according to standard procedures [20]. Two ^{32}P-labeled cDNA probes, a 1.15-kb *Kpn*I/*Eco*RI fragment and a 3.8 kb *Eco*RI/*Eco*RI fragment of p4.95BT, which recognize 5′ and 3′ sequences of the human Rb gene, respectively, were used. The probes were kindly provided by J. Rapaport, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, MA [21].

Chromosomal Analysis

The karyotype was analyzed by a standard trypsin Giemsa-banding method after the cells had been cultured for 24 hr.

RESULTS AND DISCUSSION

Chromosomal analysis of the peripheral blood cells detected a translocation between the long arm (q26.2) of chromosome 3 and the long arm (q13–14) of chromosome 13 in all cells analyzed (Fig. 1). A dicentric chromosome of chromosome 22 also was seen in about three fourths of the cells. Of the 40 cells examined, 7 showed 46,XY,t(3;13)(q26.2;q13–14); 12 showed 46,XY,t(3;13)(q26.2;q13–14), idic (22)(p11); and 21 showed 45,XY, t(3;13)(q26.2;q13–14),−7,idic(22)(p11).

Electrophoretic analysis of the RT-PCR products followed by ethidium bromide staining revealed two fragments of 250 and 280 bp in both the positive control leukemia cells and the patient's leukemia cells (data not shown). On Southern blot analysis, PCR products from the control cells and the patient's cells hybridized with the EVI1 probes (Fig. 2), confirming that the products were EVI1 gene transcripts. A recent study reported that EVI1 gene transcripts can be detected by RT-PCR in hematologic malignancies without any detectable abnormalities of chromosome 3q26 [22]. Therefore, detection of the EVI1 mRNA by Northern blot analysis is required to confirm activation of the EVI1 gene as the result of translocation. Unfortunately, we lacked a sufficient sample in order to perform Northern blot analysis. It cannot be determined whether the transcriptional activation of the EVI1 gene in our patient resulted from the chromosomal translocation.

Next, we examined expression of the Rb gene product by Western blot analysis. Whereas the amount of Rb gene product in a lymphoblastoid B-cell line established from the patient's peripheral blood was comparable to that in Daudi cells, the Rb gene product in the patient's leukemia cells was reduced substantially (Fig. 3A). The integrity of the proteins was confirmed by reprobing the filter with an anti-actin antibody (Fig. 3B). We then analyzed the Rb gene by Southern blotting to determine whether there were any gross abnormalities, such as rearrangements or deletions of the gene, as a result of the translocation. *Eco*RI, *Bam*HI, and *Hind*III were used to digest the genomic DNA. No gross abnormalities were detected in the Rb gene of the patient's leukemia cells by use of two different cDNA probes, which recognized the 5′ and 3′ regions of the gene. Comparison of intensities of the bands derived from the patient's gene and from the control gene by densitometric scans showed no difference, indicating no loss of heterozygosity.

Gross abnormalities of the Rb gene are rare in AML, even if the leukemia cells have no detectable Rb gene product [14,23]. There is no published report indicating that the Rb gene is rearranged and fused to another gene as a result of a chromosomal translocation. In chronic lymphocytic leukemia, in which chromosome 13q14 is frequently involved, an undetermined gene at 13q14, rather than the Rb gene, has been implicated [24]. From the Southern blot results, it is not likely that the Rb gene is involved in the translocation t(3;13)(q26.2;q13–14) in our patient. Recent molecular investigations have suggested that multiple genetic events, rather than a single genetic abnormality, are necessary for the development of some leukemias, such as MDS in transformation [25] and CML in blast crisis [26]. Loss of the Rb gene product combined with activation of the EVI1 gene may have been steps that led to the development of leukemia in our patient.

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